Galactose-1-phosphate Uridylyltransferase from *Escherichia coli*, a Zinc and Iron Metalloenzyme[†]

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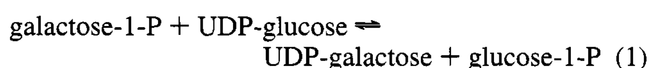
ABSTRACT: Galactose-1-P uridylyltransferase purified from *Escherichia coli* cells grown in enriched medium contains approximately 1.2 mol of tightly bound zinc/mol of subunits as well as variable amounts of iron, up to 0.7 mol/mol of subunits, and no detectable Ca, Cd, Cu, Mo, Ni, Co, Mn, As, Pb, or Se. The chelators, 1,10-phenanthroline, 8-hydroxyquinoline, 8-hydroxyquinoline sulfonate, and 2,2′-bipyridyl remove metal ions from the enzyme and allow the importance of zinc and iron to be evaluated. Dialysis of this enzyme against 2 mM 1,10-phenanthroline, 8-hydroxyquinoline sulfonate, and 2,2′-bipyridyl at millimolar concentrations slowly removes both zinc and iron from the enzyme ($t_{1/2}$ = 4 days at 24 °C) with concomitant loss of enzymatic activity. In chelation experiments utilizing 1,10-phenanthroline, residual enzymatic activity was found to be proportional to the zinc content, to the iron content, and to the sum of zinc and iron. UDP-glucose (0.35 mM) protects the enzyme against loss of metal ions and activity in the presence of 1,10-phenanthroline, whereas glucose-1-P at 70 mM (400 × K_m) fails to protect. The enzyme purified from cells grown on a minimal medium containing inorganic salts and glucose supplemented with either $ZnSO_4$ or $FeSO_4$ shows approximately the same level of enzymatic activity as the enzyme from cells grown on enriched medium. These experiments showed that enzymatic activity is supported by either iron or zinc associated with two sites in the enzyme. Enzyme depleted of metal ions by chelators can be partially reactivated by addition of $ZnSO_4$. Uridylyltransferase dialyzed against 5 M urea and EDTA is devoid of metal ions and enzymatic activity but can be reconstituted as an active metalloenzyme containing Zn(II), Fe(II), Co(II), Cd(II), or Mn(II) by further dialysis against $ZnSO_4$, $FeSO_4$, $CoCl_2$, $Cd(OAc)_2$, or $MnCl_2$. The simplest interpretation of available information is that uridylyltransferase contains two metal ion binding sites, at least one of which must be occupied to support enzymatic activity. The metal ions appear to be structural components rather than catalytic components of the enzyme.

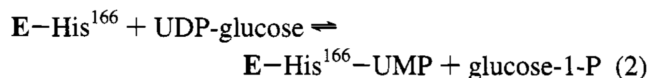
Galactose-1-phosphate uridylyltransferase (hexose-1-P uridylyltransferase, EC 2.7.7.12) catalyzes the interconversion of galactose-1-P and uridine 5′-diphosphate glucose (UDP-glucose)[1] with glucose-1-P and UDP-galactose:



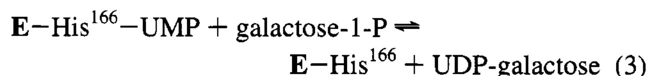
$$\text{galactose-1-P} + \text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} + \text{glucose-1-P} \quad (1)$$

Galactosemia in humans arises from any of several defects in this enzyme. The defects are inherited as autosomal recessive traits (Kalckar, 1960; Levy & Hammersen, 1978; Reichardt & Woo, 1991).

Most mechanistic studies have been carried out on the uridylyltransferase from *Escherichia coli*, a dimeric enzyme composed of identical subunits. Key aspects of the mechanism have been confirmed for the human and yeast enzymes (Frey et al., 1982; Hester & Raushel, 1987). The *E. coli* enzyme catalyzes eq 1 through a double-displacement mechanism and ping-pong kinetics according to eqs 2 and 3, in which His^{166} is the nucleophilic catalyst at the active



$$\text{E-His}^{166} + \text{UDP-glucose} \rightleftharpoons \text{E-His}^{166}\text{-UMP} + \text{glucose-1-P} \quad (2)$$



$$\text{E-His}^{166}\text{-UMP} + \text{galactose-1-P} \rightleftharpoons \text{E-His}^{166} + \text{UDP-galactose} \quad (3)$$

site (Wong & Frey, 1974a,b; Wong et al., 1977; Yang & Frey 1979; Field et al., 1989; Kim et al., 1990). Experiments by site-directed mutagenesis also led to the identification of His^{164} as an essential residue, the function of which remains unknown (Kim et al., 1990). The amino acid sequence encompassing the two essential histidine residues is FENKGAAMGCSNP**HPH**GQ; the sequence HPH is conserved in the human, yeast, and *Streptomyces* enzymes (Reichardt & Berg, 1988). This segment of the protein contains Cys^{160} and Glu^{152}, both of which are conserved in the consensus sequence for four species. Inasmuch as the side chains of histidine, cysteine, and glutamate are often the ligands for Zn^{2+} in proteins (Vallee & Auld, 1990a), the presence of these conserved residues raised the question of the possible presence of a metal ion such as Zn^{2+}. We show in this paper that galactose-1-P uridylyltransferase from *E. coli* is a metalloprotein that contains two divalent metal ions per subunit, zinc and iron, when the cells are grown on enriched medium. The metal ion composition of uridylyltransferase can be varied by growth of cells on minimal media containing variable amounts of divalent metal ions or by chelation and reconstitution. These experiments indicate that the enzyme contains two divalent metal ion binding sites

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[1] Abbreviations: UDP, uridine 5′-diphosphate; UDP-glucose, uridine 5′-diphosphate glucose; UDP-galactose, uridine 5′-diphosphate galactose; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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per subunit and that one or both must be occupied to support enzymatic activity.

MATERIALS AND METHODS

Materials. 1,10-Phenanthroline monohydrate was obtained from GFS Chemicals. 8-Hydroxyquinoline, 8-hydroxyquinoline sulfonate, and 2,2′-bipyridyl were obtained from Aldrich Chemical Co. BICINE and HEPES (Ultrol grade) were obtained from Sigma and Calbiochem, respectively. The following chelators were further purified by recrystallization (2×): 1,10 phenanthroline from 95% aqueous ethanol and 8-hydroxyquinoline from ethanol.

Cell Culture. *E. coli* BL21 cells transformed with plasmid pTLC5800 (Field et al., 1989) were grown at 37 °C from seed stocks, either in 2× YT medium (16 g of Bacto tryptone, 10 g of Bacto yeast extract, and 5 g of NaCl per liter of distilled water) or in minimal medium, M9 (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 0.5 g of NaCl, 1.0 g of NH_4Cl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.018 g of $CaCl_2 \cdot 2H_2O$, and 4 g of glucose per liter of distilled water), both containing 100 μg/mL ampicillin. Cells were allowed to grow to a density of approximately 1 OD at 600 nm after which the inducer IPTG was added to a concentration of 1 mM. After an additional 3 h of culture, cells were harvested by centrifugation at 12 000*g*, frozen in liquid nitrogen, and stored at −135 °C.

Purification of Galactose-1-P Uridylyltransferase. Galactose-1-P uridylyltransferase from *E. coli* was purified by the method of Arabshahi et al. (1986) with the following changes. The enzyme was overexpressed in *E. coli* BL21 cells transformed with plasmid pTLC5800 (Field et al., 1989). All buffers were scrubbed of metal ions either by dithizone extraction or by treatment with Chelex 100 (Holmquist, 1988) and contained 10 mM β-mercaptoethanol. Desalting steps utilized either Sephadex G25 column chromatography or two dialyses of 2.5 h duration. In the last chromatographic step, Q-Sepharose Fast Flow was substituted for DEAE-Sephadex A-50 and a linear salt gradient was instituted consisting of 1 L each of 0.05 M NaCl and 0.3 M NaCl in 0.01 M HEPES buffer at pH 7.5. In addition, the purified enzyme was concentrated in an Amicon Ultrafiltration stirred cell prior to drop-freezing in liquid nitrogen. The frozen enzyme was stored at −135 °C. Protein homogeneity was established by amino acid analysis which agreed within ± 5% with the expected analysis based on DNA sequence data of the *gal*T gene (Lemaire & Muller-Hill, 1986; Cornwell et al., 1987).

Assay of Galactose-1-P Uridylyltransferase. Enzymatic activity was measured by use of the standard coupled assay described by Wong and Frey (1974b). The specific activity of preparations used for these studies ranged from 170 to 190 units/mg of protein.

Metal Analysis. Preliminary metal analyses were conducted by inductively coupled plasma emission spectroscopy, the results of which indicated the presence of both zinc and iron, but no other metals, in all samples of the enzyme. Subsequently, samples were analyzed for zinc and iron by graphite furnace atomic absorption spectrometry.

Protein Analysis. Protein concentrations of analyzed samples were measured by amino acid analysis using the Pico Tag system of Waters Associates and were based on the expected amino acid composition of isoleucine, leucine, and phenylalanine (I + L + F = 52) expressed as residues per subunit of enzyme, which is known from the nucleotide sequence of the *gal*T gene in *E. coli* (Lemaire & Muller-Hill, 1986; Cornwell et al., 1987). The extinction coefficient at 280 nm for galactose-1-P uridylyltransferase monomer was calculated from the A_{280} measurements on samples of four enzyme preparations, the molar concentrations of which had been established by amino acid analysis. The value of ϵ_{280} was found to be (7.24 ± 0.24) x 10^4 M^{-1} cm^{-1}.

Metal Chelation. Metal chelation studies were conducted by dialysis of the enzyme (40−50 μM subunits) at either 4 or 24 °C against 2000 volumes of buffer (0.05 M HEPES at pH 7.5 or 8.0, 0.5 M NaCl, and 10 mM β-mercaptoethanol) containing various chelators. Samples evaluated for metal content were subsequently dialyzed against metal-scrubbed buffer for 2 days at 4 °C (Auld, 1988a). Enzyme treated with salts of either Zn(II) or Fe(II) or both were dialyzed against 1000 volumes of buffer containing 100 μM of each metal salt for 2 days at 24 °C and then dialyzed against buffer alone for an additional 2 days at 4 °C. Metal ion reconstitution was conducted inside a Coy anaerobic chamber which contained no detectable dioxygen (<1 ppm). All dialyses were conducted by use of 1 cm diameter dialysis tubing (Spectrapor-2).

RESULTS

Metal Analyses. Initial analyses of four highly purified but not homogeneous preparations of galactose-1-P uridylyltransferase by inductively coupled plasma emission spectroscopy indicated the presence of approximately equivalent amounts of zinc and iron. The average zinc content was 0.72 ± 0.24 (SD) mol/mol of dimer, and the average iron content was 0.73 ± 0.24 (SD) mol/mol of dimer. The concentration of enzyme in these samples was estimated from measurements of A_{280} assuming an extinction coefficient of 72 400 M^{-1} cm^{-1}. These initial preparations exhibited a major band upon gel electrophoresis, which migrated at a rate corresponding to galactose-1-P uridylyltransferase, and a number of trace impurities. These initial analyses by inductively coupled plasma emission spectroscopy ruled out the presence of Cd, Cu, Mo, Ni, Co, Mn, As, Pb, and Se in galactose-1-P uridylyltransferase. The consistent presence of both iron and zinc in substantial and approximately equimolar amounts in the initial analyses suggested that the uridylyltransferase may be a zinc−iron metalloprotein. The metals were unlikely to be associated only with trace impurities because of the amounts found and the fact that there were no major impurities.

To obtain accurate values for the zinc and iron content of galactose-1-P uridylyltransferase, we purified six additional samples under carefully controlled conditions by making use of buffers freed of metal ions. We verified the purities of these preparations by quantitative amino acid analyses and obtained the results shown in Table 1. The amino acid content corresponded closely with the amino acid composition of this protein, as deduced from the amino acid sequence derived by translation of the nucleotide sequence of the gene. The analysis is also similar to that reported by Saito et al. (1967). The purity of four samples was estimated to be >95% based on their amino acid content.

The amounts of zinc and iron associated with the uridylyltransferase in six preparations that had been purified by use of buffers freed of divalent metal ions are given in Table 2. The enzyme contains more than 1 mol of zinc and less than 1 mol of iron per mole of subunits, and the combined

Table 1: Amino Acid Composition of Galactose-1-P Uridylyltransferase from *E. coli*

amino acid	residues per subunit	
	expected[a]	found ± SD[b]
Asp + Asn	31	29.3 ± 3.8
Glu + Gln	42	42.8 ± 1.6
Ser	19	16.4 ± 2.9
Gly	16	15.7 ± 1.7
His	15	15.1 ± 0.7
Arg	21	21.0 ± 1.0
Thr	23	22.1 ± 1.0
Ala	33	32.9 ± 1.0
Pro	26	26.5 ± 1.0
Tyr	11	11.0 ± 0.3
Val	22	20.1 ± 0.2
Met	8	7.4 ± 1.1
Ile	6	5.5 ± 0.4
Leu	31	31.6 ± 0.4
Phe	15	15.1 ± 0.1
Lys	13	13.1 ± 1.0

[a] Amino acid composition based on the amino acid sequence. [b] Average composition from analysis obtained for four preparations of the enzyme.

Table 2: Zinc and Iron Content of Galactose-1-P Uridylyltransferase

preparation	activity	Zn (mol/mol)[a]	Fe (mol/mol)[a]
1	167	1.20	0.84
2		1.20	0.56
3	174	1.19	0.62
4	188	1.38	0.86
5	194	1.10	0.56
6	183	1.19	0.59
mean ± SD	181 ± 10	1.21 ± 0.09	0.67 ± 0.14

[a] Moles per mole of enzyme subunits.

iron and zinc contents correspond very nearly to two metal ions per subunit. The ratios of the two metal ions and their respective contents per milligram of protein remained constant within error in individual fractions collected in the last column purification step.

Effects of the Metallic Composition of Growth Media on the Uridylyltransferase. The enzyme samples in Table 2 had been purified from cells grown in an enriched medium (2x YT) containing approximately 20 μM Zn and 20 μM Fe. Further studies were aimed at determining whether the metallic content of the growth medium would influence the metal ion composition of the purified enzyme. Experiments were conducted to determine how *E. coli* BL21 transformed with pTLC5800 would express the uridylyltransferase when grown on a more stringent growth medium. We chose M9, a minimal essential medium containing sodium and potassium phosphates, NaCl, $MgSO_4$, $CaCl_2$, and NH_4Cl as a nitrogen source, and glucose as a carbon source. Salts of other divalent metal ions are normally not added because they are present at low levels in the M9 salts. Cells cultured at 37 °C in M9 medium or in M9 supplemented with $ZnSO_4$ were found to grow slower ($t_{1/2}$ = 80 min) than in enriched (2x YT) medium ($t_{1/2}$ = 35 min). Cells grown in M9 media with and without supplementation with $ZnSO_4$ were harvested, lysed by sonication, and centrifuged, and the supernatant fluids were assayed for enzymatic activity. Minimal medium M9 contained approximately 0.6 μM Zn and 2 μM Fe. Galactose-1-P uridylyltransferase activity (units per milligram of protein) increased approximately 3-fold in the extracts when the concentration of Zn^{2+} was increased to 10 μM or more in the medium (Figure 1A). Increased activity upon Zn supplementation could have resulted from increased uridylyltransferase synthesis or enhanced enzymatic activity owing to greater incorporation of Zn into the enzyme. The results of polyacrylamide gel electrophoresis of cellular extracts supported the former interpretation (Figure 1B). Although equal amounts of protein in the cellular extracts were applied in each lane, the intensity of the stained band representing the uridylyltransferase increased with the $ZnSO_4$ concentration in the culture medium.

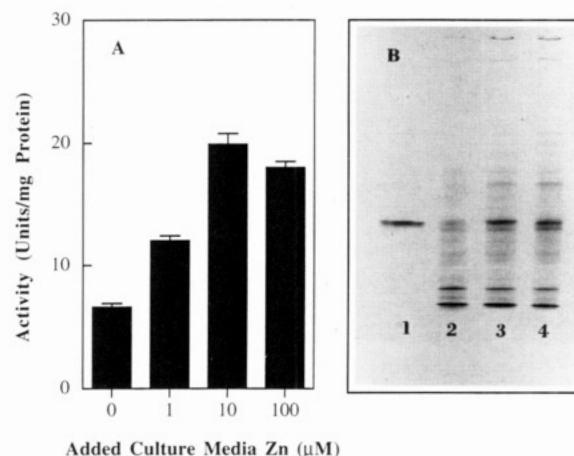


FIGURE 1: Galactose-1-P uridylyltransferase expression in *E. coli* following zinc supplementation of culture medium (M9). *E. coli* cells (BL21 DE3) containing the plasmid pTLC5800 (Field et al., 1989) were grown at 37 °C in minimal media (M9) (see Materials and Methods) supplemented with 0, 1, 10, or 100 μM $ZnSO_4$, 100 μg/mL ampicillin, and 1 mM IPTG. Harvested cells were broken by sonication and cell extracts prepared by centrifugation at 40 000*g* for 30 min at 4 °C. (A) Enzyme activity according to the standard assay (see Materials and Methods); protein measured by UV absorption at 280 and 260 nm (Layne, 1957). (B) Native polyacrylamide gel electrophoresis (8–25% gel) by the Phastgel System (Pharmacia). Lane 1, purified galactose-1-P uridylyltransferase; lane 2, extract from cells grown on minimal medium M9; lane 3, extract from cells grown on M9 + 10 μM $ZnSO_4$; lane 4, extract from cells grown on M9 + 100 μM $ZnSO_4$. Equal amounts of protein (5 μg) were applied in each lane.

Galactose-1-P uridylyltransferase is expressed at high levels in cells grown in a minimal medium that contains a low concentration of Fe (2 μM) but is supplemented with either 10 or 20 μM Zn; the enzyme purified from these cells contains slightly more than one zinc per subunit but is depleted in iron (experiments 2 and 6, Table 3). The specific activity and zinc content per subunit are similar to those of enzyme purified from cells grown in enriched medium. However, the iron content is less than one-third of that in enzyme purified from cells grown in the enriched medium. Uridylyltransferase purified from cells grown in minimal medium supplemented with 20 μM Fe and containing only 0.8 μM Zn still contains 0.85 zinc per subunit, as well as 1.5 irons per subunit, and is approximately 85% as active as the enzyme purified from cells grown in enriched medium (experiment 4, Table 3). That is, almost the full complement of zinc was present in the purified enzyme even though the zinc concentration in the medium was only $^1/_{25}$th of that in either the enriched or the zinc-supplemented medium (experiments 1 and 2 of Table 3).

Experiments 3 and 5 of Table 3 show that, under forcing conditions of very high zinc or iron supplementation in minimal cell growth media, galactose-1-P uridylyltransferase can be produced that contains either 2 mol of zinc or 2 mol of iron per subunit. The specific activity depends very little

Table 3: Effect of Metal Content of Cell Culture Media on the Zinc and Iron Contents of Galactose-1-P Uridylyltransferase

growth medium	enzyme Zn (mol/mol)[a]	enzyme Fe (mol/mol)[a]	Zn + Fe (mol/mol)[a]	specific activity[b]	medium analysis Zn(μM)	Fe(μM)
(1) enriched medium (2× YT)	1.19 ± 0.13[c]	0.59 ± 0.04	1.78	168 ± 2	22	21
(2) M9 + 20 μM $ZnSO_4$	1.09 ± 0.07	0.23 ± 0.01	1.32	174 ± 6	21	3
(3) M9 + 100 μM $ZnSO_4$	2.07 ± 0.12	0.21 ± 0.04	2.28	175 ± 7	96	3
(4) M9 + 20 μM $FeSO_4$	0.85 ± 0.04	1.47 ± 0.05	2.32	149 ± 3	0.8	22
(5) M9 + 200 μM $FeSO_4$	0.12 ± 0.01	2.01 ± 0.11	2.13	197 ± 12	1	196
(6) M9 + 10 μM $ZnSO_4$	1.21 ± 0.05	0.08 ± 0.01	1.29	150 ± 3	8	2

[a] Metal content per mole of enzyme subunits. [b] Units per milligram of protein (±SD in quadruplicate assays). [c] MEAN ± SD.

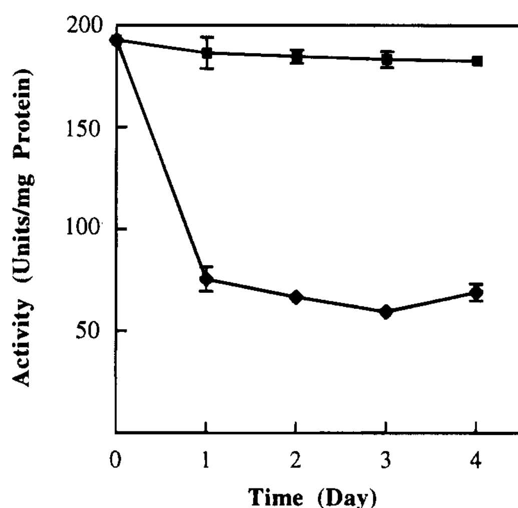


FIGURE 2: Instability of iron-containing uridylyltransferase during dialysis. Samples of iron–uridylyltransferase (40 μM) from experiment 5 of Table 3, which had been purified at 4 °C, were dialyzed against 0.05 M HEPES buffer at pH 7.5 containing 0.05 M NaCl and 10 mM β-mercaptoethanol at 24 °C inside the anaerobic chamber in the presence and absence of 0.35 mM UDP-glucose. Aliquots were removed at the indicated times, quenched by diluting into ice-cold buffer, and assayed for enzymatic activity. Symbols: (■) enzyme plus UDP-glucose; (♦) enzyme alone. In the latter experiment, the iron content decreased to 0.85 mol/mol in the course of dialysis, and the activity decreased to 30% of its initial value.

on whether the metal ions are zinc or iron, and the amounts of enzyme purified from the cells are comparable. However, the iron-containing enzyme in experiment 5 of Table 3 is less stable toward dialysis at ambient temperature than the zinc-containing enzyme, as shown by the experiment of Figure 2. Both enzymes can be purified at low temperature by the usual procedure and are equally active, but the enzyme containing two irons per subunit and very little zinc quickly loses its activity upon dialysis at 24 °C, whereas the zinc-containing enzyme retains its activity for days when subjected to dialysis. Inactivation of the iron enzyme cannot have resulted from air-oxidation of Fe(II) because the dialysis was carried out under anaerobic conditions.

Metal Ion Chelation Studies. Chelating agents can often remove divalent metal ions from proteins (Wagner, 1988; Auld, 1988b). The chelation experiments in Figures 3–5 demonstrate a dependence of enzymatic activity on the metal ion content of galactose-1-P uridylyltransferase. 1,10-Phenanthroline removes zinc (Figure 3A) and inactivates the enzyme (Figure 3B) in a first-order and chelator concentration-dependent process in the course of 1 week. The half-times for the loss of enzyme activity in the presence of 1,10-phenanthroline at 24 °C are 4.1 ± 1 days at 5 mM and 15 ± 1 days at 2 mM 1,10-phenanthroline, respectively. The half-times for the loss of zinc are 4.0 ± 1 days at 5 mM and 14 ± 1 days at 2 mM 1,10-phenanthroline, respectively. The

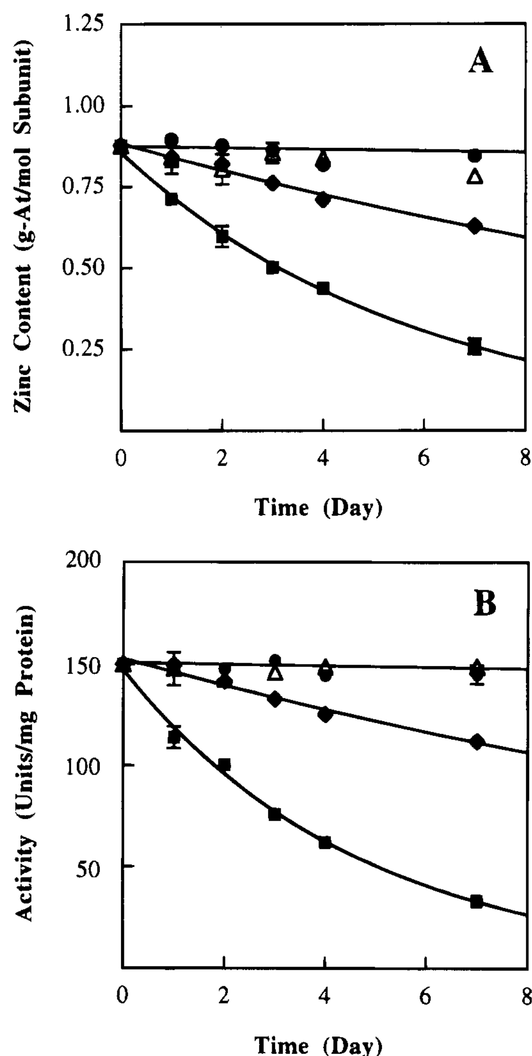


FIGURE 3: Concentration-dependent loss of Zn and galactose-1-P uridylyltransferase activity by 1,10-phenanthroline. Galactose-1-P uridylyltransferase purified from cells grown on enriched medium contained both iron and zinc. The enzyme (40 μM) was dialyzed against 0, 0.5, 2, or 5 mM 1,10-phenanthroline (500 volumes) in HEPES buffer (0.05 M, pH 8.0) containing 0.05 M NaCl and 10 mM β-mercaptoethanol 24 °C. At selected times, enzyme samples were removed and dialyzed against HEPES buffer for 48 h at 4 °C to remove the chelator. Aliquots were assayed for enzymatic activity and for zinc by graphite furnace atomic absorption spectrophotometry. Part A: Zn content versus time. Part B: Enzyme activity versus time. Symbols: (●) no chelator; (△) 0.5 mM 1,10-phenanthroline; (♦) 2 mM 1,10-phenanthroline; (■) 5 mM 1,10-phenanthroline.

uridylyl-donor substrate UDP-glucose prevents the loss of zinc and iron and enzymatic activity, as shown in Figure 4A,B, which also shows that the half-times for the extraction of iron and zinc by 1,10-phenanthroline in the absence of UDP-glucose are the same at 33 °C. Glucose-1-P at a

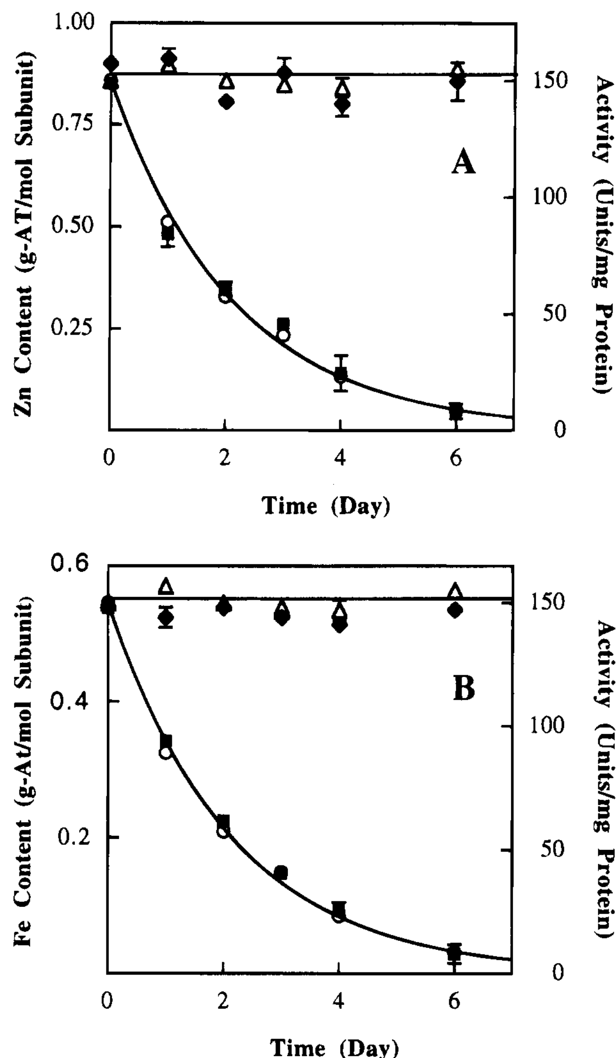


FIGURE 4: Protection by UDP-glucose against the extraction of metal ions and inactivation of galactose-1-P uridylyltransferase activity by 1,10-phenanthroline. Galactose-1-P uridylyltransferase purified from cells grown on enriched medium contained both iron and zinc. Enzyme (40 μM) was treated with 5 mM 1,10-phenanthroline at pH 7.5 (see Figure 3 for procedure) in the presence and absence of 0.35 mM UDP-glucose at 33 °C. Part A: (○) zinc content; (■) enzymatic activity; (♦) zinc content in the presence of UDP-glucose; (△), enzymatic activity in the presence of UDP-glucose. Part B: (○) iron content; (■) enzymatic activity; (♦) iron content in the presence of UDP-glucose; (△) enzymatic activity in the presence of UDP-glucose.

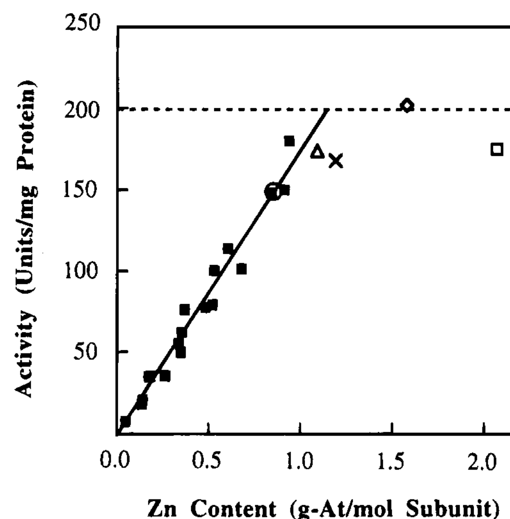


FIGURE 5: Relationship between Zn^{2+} content and galactose-1-P uridylyltransferase activity. Galactose-1-P uridylyltransferase purified from cells grown on enriched medium contained both iron and zinc. Samples of this enzyme were treated in various ways and assayed for zinc content and enzymatic activity. Symbols: (■) enzyme was treated with 1,10-phenanthroline (5 mM) at 4, 24, or 33 °C for varying times by dialysis in HEPES buffer and analyzed for zinc and enzymatic activity (see Figure 3 for procedure); (◇) uridylyltransferase supplemented with Zn(II) by dialysis against HEPES buffer containing 0.05 M NaCl, 10 mM β-mercaptoethanol, and 100 μM $ZnSO_4$ for 48 h at 24 °C and then against HEPES buffer at 4 °C to remove excess $ZnSO_4$; (×) uridylyltransferase purified from cells grown in (2× YT) culture medium (experiment 1 in Table 3); (△) uridylyltransferase purified from cells grown in medium M9 supplemented with 20 μM $ZnSO_4$ (experiment 2, Table 3); (□) uridylyltransferase purified from cells grown in medium M9 supplemented with 100 μM $ZnSO_4$ (experiment 3, Table 3); (○) uridylyltransferase purified from cells grown in medium M9 supplemented with 20 μM Fe (experiment 4, Table 3).

concentration of 70 mM, over 400 times the K_m and 3 times the substrate inhibition constant (Wong & Frey, 1974b), does not affect the rate of zinc removal or loss of enzymatic activity (data not shown).

In Figure 5, data on the zinc content of various uridylyltransferase samples from the experiments in Table 3 and Figure 3A,B are plotted versus their enzymatic activities. The enzymatic activity is directly proportional to the zinc content and increases to a maximum of about 200 units/mg of protein, as reported previously for this enzyme (Wong & Frey, 1974b). Maximum activity is attained at about 1.1 mol of zinc/mol of enzyme subunits. In addition, incubation of the untreated enzyme at 40 μM with $ZnSO_4$ at 100 μM for 2 days at 24 °C increases enzymatic activity by about 15% to the maximum of 200 units/mg, and leads to a zinc content of 1.6 mol/mol of subunit and an iron content of 0.6 mol/mol of subunit after extended dialysis to remove excess $ZnSO_4$. The correlation in Figure 5 documents the proportionality of zinc content with enzymatic activity and that at least one zinc per subunit is required for activity. However, analogous plots of iron plus zinc are similarly linear (data not shown), and the maximum activity is attained at a stoichiometry of about two metal ions per subunit. Therefore, Figure 5 does not mean that only one divalent metal ion per subunit is essential for activity because all of the samples preceding the plateau in Figure 5 contained iron as well as zinc. The open square in the plateau corresponds to a sample that contained two zinc ions per subunit and a decreased iron content. The important point from Figure 5 is that the enzymatic activity is dependent on the presence of zinc in one or both sites. We will show in a later section that activity is supported by any of several divalent metal ions in both sites. Excess, adventitiously bound zinc resulting from incubation of the enzyme with $ZnSO_4$ neither increases nor inhibits enzymatic activity; however, excess adventitiously bound iron resulting from incubation of the enzyme with $FeSO_4$ inhibits enzymatic activity (data not shown).

Chelators other than 1,10-phenanthroline also remove divalent metal ions from the uridylyltransferase. Alternative chelators are 2 mM 8-hydroxyquinoline sulfonate and 2 mM 2,2′-bipyridyl, which extract zinc and iron commensurate with loss of enzymatic activity similar to 1,10-phenanthroline. In contrast, 8-hydroxyquinoline preferentially extracts iron. Dialysis of 40 μM enzyme against 5 mM 8-hydroxyquinoline in a buffer (50 mM HEPES at pH 7.5, 50 mM NaCl, and 10 mM β-mercaptoethanol) for 2 days at 24 °C inside an anaerobic chamber reduced the iron content of the enzyme

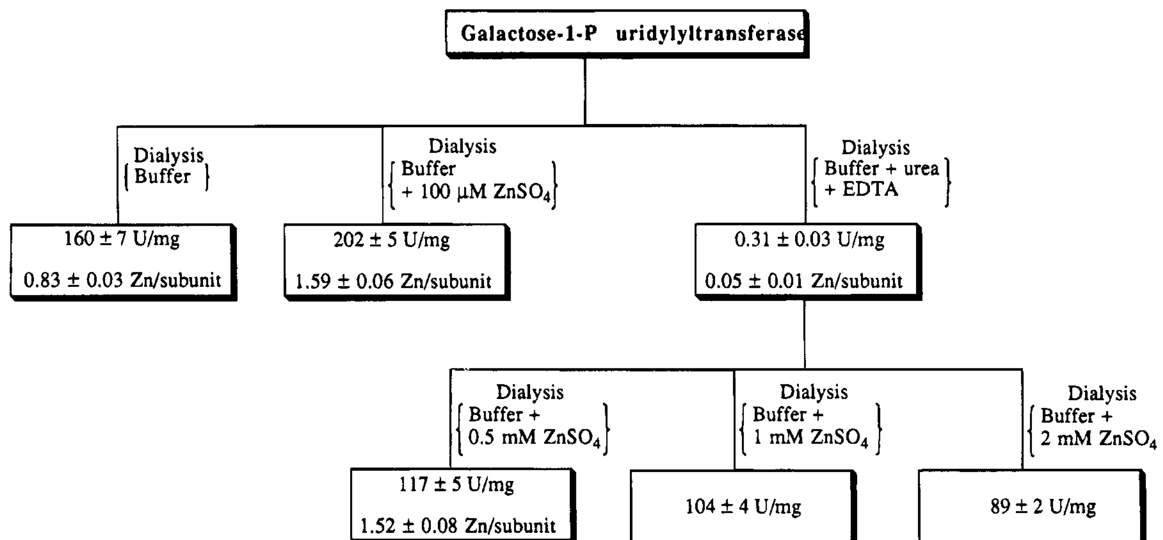


FIGURE 6: Flow chart for reconstitution of enzymatic activity in metal-depleted galactose-1-P uridylyltransferase by $ZnSO_4$. Three samples of galactose-1-P uridylyltransferase (40 μM, 1 mL) were initially dialyzed against solutions containing buffer (0.5 L, 48 h at 24 °C), buffer plus 100 μM $ZnSO_4$ (0.5 L, 48 h at 24 °C and then against buffer alone at 4 °C for 48 h), or buffer containing 250 μM EDTA and 5 M urea (50 mL, 16 h at 24 °C). The buffer consisted of 0.05 M HEPES buffer at pH 8.0 containing 0.05 M NaCl and 10 mM β-mercaptoethanol. The samples were assayed for enzymatic activity and zinc content with the results indicated. The zinc-depleted sample was again dialyzed, first against 50 mL of buffer containing 5 M urea and 0.5, 1, or 2 mM $ZnSO_4$ for 24 h at 24 °C, then against 50 mL of buffer containing 0.5, 1, or 2 mM $ZnSO_4$ for 24 h at 24 °C, and finally against 0.5 L (one change) of buffer alone for 48 h at 4 °C.

from 0.51 to 0.03 mol/mol of subunit and the zinc from 1.16 to 0.99 mol/mol of subunit. Although 94% of the iron was removed, only 15% of the zinc was removed, and the activity decreased to 55% of its original value. It is clear from this experiment that the enzyme does not specifically require iron and significant activity is supported by the zinc remaining after treatment with 8-hydroxyquinoline. The decreased activity may represent partial occupancy of both sites by zinc or that 8-hydroxyquinoline reacts in some manner other than chelation to inhibit its activity.

Experiments To Reconstitute Divalent Metal Ions in Uridylyltransferase. Preliminary reconstitution experiments showed that simple dialysis of $ZnSO_4$ into solutions of uridylyltransferase that had been depleted of divalent metal ions by dialysis against a chelator invariably increases the residual enzymatic activity by 30−50%, whereas dialysis of the same samples against $FeSO_4$ invariably fails to increase the activity and actually decreases it. In no case did dialysis against $ZnSO_4$ fully reconstitute activity; moreover, this treatment led to the binding of excess zinc by the enzyme. Dialysis of metal-depleted enzyme against a mixture of $ZnSO_4$ and $FeSO_4$ restored activity to levels no greater than that attained by dialysis against $ZnSO_4$ alone.

In order to test other divalent metal ions for their ability to substitute for zinc or iron, we undertook to devise an improved reconstitution procedure that would remove essentially all of the metal ions and then allow them to be replaced with substantial recovery of enzymatic activity. The procedures described below resulted from this effort.

EDTA is not an effective chelator of the metal ions bound to galactose-1-P uridylyltransferase in the absence of a denaturant (data not shown). However, at increasing concentrations of urea in the presence of EDTA, significant enzymatic activity is lost within 4 h at 24 °C. The activity loss corresponds to decreased zinc content. In several experiments, the urea concentration was varied at 0.1 mM EDTA (buffer: 50 mM HEPES, pH 8.0, 10 mM β-mercaptoethanol, 24 °C). The solutions were placed at 24 °C for 4 h, dialyzed against the buffer for 16 h (±0.1 mM $ZnSO_4$) to remove the urea, and assayed for zinc and activity. The following zinc contents (moles per mol of subunit, mean ± SD) were measured for each urea concentration: no urea, 0.87 ± 0.03; 1 M urea, 0.86 ± 0.07; 2 M urea, 0.75 ± 0.02; 3 M urea, 0.70 ± 0.06; 5 M urea, 0.35 ± 0.01. The loss of enzymatic activity was substantial at 5 M urea (>95%) but the activity was significantly protected in all the experiments by the presence of 0.1 mM $ZnSO_4$ in the urea and dialysis buffers. Even at 5 M urea, approximately 50% of the activity of the untreated control was recovered in the enzyme treated with both urea and $ZnSO_4$.

The flow chart in Figure 6 summarizes the results of a study designed to find reconstitution conditions for uridylyltransferase that had been depleted of zinc by treatment with EDTA in the presence of 5 M urea. Dialysis of the uridylyltransferase against 1 mM EDTA in 5 M urea removed essentially all of the zinc and reduced its activity to 0.3% of normal. Continued dialysis, first against 0.5 mM $ZnSO_4$ and 5 M urea and then against 0.5 mM $ZnSO_4$, restored the enzymatic activity to 73% of its original value. Reconstitution at higher concentrations of $ZnSO_4$ restored activity to a lesser extent.

The zinc reconstitution procedure of Figure 6 also allows other divalent metal ions to be tested for their ability to activate the uridylyltransferase. Table 4 shows that the zinc- and iron-depleted enzyme is activated by $CoCl_2$, Cadmium acetate, $FeSO_4$, and $MnCl_2$ to 40−50% of the activity of the Zn-reconsituted enzyme.

DISCUSSION

In the most straightforward interpretation of the present results, we can conclude that galactose-1-P uridylytransferase from *E. coli* is a metalloprotein containing two divalent metal ions per subunit. Although its metal ion composition can be influenced by the metal ion composition of the cellular growth medium, one site tends to be occupied preferentially by zinc and the other by iron, although both sites can be occupied by either metal ion. That the metal ions exhibit

Table 4: Reconstitution of Metal-Depleted Galactose-1-P Uridylyltransferase with Divalent Metal Ions

form of enzyme	specific activity (units/mg of protein)	zinc (mol/mol)[a]	Me(II) (mol/mol)[a]	iron (mol/mol)[a]
native (Zn/Fe)	168 ± 2	1.19 ± 0.13		0.59 ± 0.04
urea/EDTA treated	0.028 ± 0.004	0.011 ± 0.002		0.026 ± 0.001
Zn(II) reconstituted[b]	95.2 ± 4.8	1.64 ± 0.08	1.64 ± 0.08	0.011 ± 0.001
Co(II) reconstituted	65.7 ± 2.3	0.012 ± 0.003	1.44 ± 0.05	0.052 ± 0.012
Cd(II) reconstituted	51.5 ± 3.5	0.035 ± 0.006	1.11 ± 0.02	0.020 ± 0.004
Fe(II) reconstituted	49.2 ± 2.2	0.070 ± 0.02	1.68 ± 0.07	1.68 ± 0.07
Mn(II) reconstituted	44.2 ± 2.1	0.075 ± 0.013	1.32 ± 0.13	0.012 ± 0.004

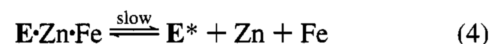
[a] Moles per mole of subunits. [b] Galactose-1-P uridylyltransferase (10 mL, 40 μM) was dialyzed against 1 L of 5 M urea in HEPES buffer at pH 7.5 containing 0.05 M NaCl, 0.25 mM EDTA, and 10 mM β-mercaptoethanol for 24 h at 24 °C. Aliquots (1 mL) were dialyzed at 24 °C for 24 h against 150 mL of buffer minus EDTA plus the following salts at 500 μM: Zn(II) sulfate, Cd(II) acetate, $FeSO_4$, $CoCl_2$, and $MnCl_2$. The solutions were dialyzed against 150 mL of the metal-buffer minus urea and then against 1 L of 0.05 M HEPES at pH 7.5 containing 0.05 M NaCl, 0.25 M EDTA, and 10 mM β-mercaptoethanol at 4 °C for 24 h (one buffer change). All dialyses were carried out inside the anaerobic chamber.

preferences for binding to different sites is supported by the fact that part of the iron is easily removed by dialysis in the absence of a chelator from enzyme that contains iron in both sites (Figure 2) and by the difficulty of obtaining enzyme that lacks zinc. To substantially deplete the enzyme of zinc, we must grow bacteria in a minimal medium supplemented with 200 μM $FeSO_4$, whereas to obtain iron-depleted enzyme we need only 20 μM $ZnSO_4$ with minimal medium (Table 3). Neither metal ion is easily removed from its preferential binding site by ordinary dialysis, but both can be removed by dialysis for a week against any of several chelators or against EDTA in the presence of 5 M urea. The metal ion depleted enzyme is inactive, and the residual activity is proportional to the amount of metal that remains, which shows that at least one of the divalent metal ions is essential for activity. Activity can be substantially reconstituted in the metal ion depleted enzyme by dialysis against divalent metal-ion salts, such as $ZnSO_4$, $FeSO_4$, $CoCl_2$, $MnCl_2$, and cadmium acetate. Therefore, although enzymatic activity requires a divalent metal ion and Zn(II) is preferred, the enzyme is not absolutely specific for Zn(II) in its preferential site.

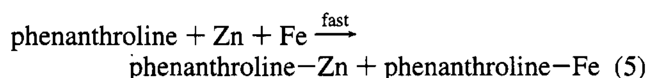
At least one divalent metal ion is required for enzymatic activity, and both metal ions may be required. Prolonged dialysis against 8-hydroxyquinoline removes nearly all of the iron from its binding site and very little zinc from its preferential site, and the enzyme retains 55% of its activity. The residual activity may represent partial occupancy of both sites by zinc. Although 8-hydroxyquinoline selectively removes iron from the enzyme, the stability constants for the Zn(II) and Fe(II) chelates of 8-hydroxyquinoline are nearly identical (Auld, 1988b). Inasmuch as 1,10-phenanthroline extracts the two metal ions at the same rate (Figure 4), it seems that 8-hydroxyquinoline may extract iron selectively by a mechanism that entails its interaction with the enzyme.

The enzyme must bind zinc and iron very tightly for them to be present at nearly stoichiometric levels after several days of chromatographic purification in metal-scrubbed buffers. Studies with several chelators support this conclusion. Typical half-times for removal of zinc and iron are approximately 4 days at 24 °C and 16 days at 4 °C (data not shown) in the presence of 5 mM 1,10-phenanthroline, indicating that the metal ions do not freely diffuse into the medium. Galactose-1-P uridylyltransferase differs in this respect from several zinc-containing proteins, which relinquish their metal ions to this chelator with half-times of minutes to hours (Haeggstrom et al., 1990; Stocker et al., 1988).

The presence of UDP-glucose, which converts galactose-1-P into its uridylyl-enzyme form (eq 2), prevents the removal of zinc by 1,10-phenanthroline and maintains enzymatic activity. Therefore, the uridylyl-enzyme must bind zinc more tightly than the free enzyme. This may be because the UMP group is a ligand for Zn(II) in the uridylyl-enzyme or because the uridylyl-enzyme is more tightly folded and does not relinquish Zn(II) to a chelator as readily as the free enzyme. The fact that both iron and zinc are removed by 1,10-phenanthroline at the same rate suggests that the latter may be the correct explanation. The simplest interpretation of equal rates is that the rate of metal ion extraction is governed by some process other than chelation *per se*. If the metal ions were released from the enzyme as a result of a reversible conformational change, the chelator would capture them as free metal ions at the same rate. This is described by eqs 4 and 5, in which the equilibrium constant



$$\mathrm{E{\cdot}Zn{\cdot}Fe} \overset{\text{slow}}{\rightleftharpoons} \mathrm{E^* + Zn + Fe} \quad (4)$$



$$\text{phenanthroline} + \mathrm{Zn} + \mathrm{Fe} \xrightarrow{\text{fast}} \text{phenanthroline}{-}\mathrm{Zn} + \text{phenanthroline}{-}\mathrm{Fe} \quad (5)$$

for the conformational change of E into E* in eq 4 is unfavorable but allows both zinc and iron to escape from the enzyme at a very slow rate. In the absence of a chelator other than the enzyme, the metal ions would be recaptured by reversal of eq 4. 1,10-Phenanthroline can capture the free metal ions according to eq 5. UDP-glucose would inhibit this process by transforming the enzyme into the uridylyl-enzyme according to eq 2, which presumably does not undergo the conformational change of eq 4. This mechanism of chelation does not exclude the UDP-glucose or the UMP-moiety of the uridylyl-enzyme as metal ion ligands. However, although we know that enzymatic ligands bind the metal ions, we have no evidence for coordination of the substrate with metal ions.

The present results do not provide information regarding the locations of metal ions in the protein. Amino acid residues in the vicinity of the active site offer good candidates for zinc ligands, including the side chains of His^{164} and His^{166}, Cys^{160} and Glu^{152}. In other Zn(II) proteins, three or four ligands are amino acid side chains. When Zn(II) is bound by four protein ligands, they are generally either all cysteine side chains or two or three cysteines and one or two histidine side chains. In these cases, Zn(II) is generally thought to stabilize the active protein structure as distinguished from participating directly in a catalytic or other functional role. When Zn(II) has three protein ligands, they are generally

selected from the side chains of histidine, cysteine, and glutamate, with water as the fourth ligand (Vallee & Auld, 1990b). In these cases, the protein is generally an enzyme, and Zn(II) is thought to participate in catalysis, although it is no doubt also important in maintaining structure in the active site.

Several enzymes have Zn(II) ligands donated by two histidines that are separated by one or three other amino acids in the sequence (Vallee & Auld, 1990a). For example, in carbonic anhydrase, two histidines in the active site are separated by phenylalanine, and in thermolysins, they are separated by glutamate and two other amino acids. Galactose-1-P uridylyltransferase from *E. coli* contains two sequences of HPH, the active site-residues $H_{164}P_{165}H_{166}$ and the nonessential histidines in the sequence $H_9P_{10}H_{11}$. The latter sequence is flanked by an aspartate at position eight. Either or both sequences may be considered as possible sources of side chain ligands for metal ions. Moreover, the protein contains six cysteine residues, including Cys^{52} and Cys^{55}, in addition to Cys^{160}, that could participate in binding metal ions. With these residues, and a cluster of acidic residues in the sequence $E_{(178)}$AEREDRLQKEYFAE$_{(192)}$, there are many possibilities for binding metal ions to this protein.

The finding of Zn in uridylyltransferase places it among the Zn-containing class II nucleotidyl transferase enzymes, which include DNA polymerase (Slater et al., 1971; Springgate et al., 1973), RNA-dependent DNA polymerase (Coleman, 1974; Auld et al., 1974), and RNA polymerase (Scrutton et al., 1971). Although stoichiometric amounts of Zn have been found, it is not clear what role it plays in these enzymes. In fact, in the case of DNA polymerase I from *E. coli* and T7 RNA polymerase, enzymes fully active for DNA polymerization or transcription but devoid of Zn have been purified (Walton et al., 1982; King et al., 1986). DNA polymerase from *E. coli* is a multifunctional enzyme that catalyzes not only DNA polymerization but also 3′- and 5′-exonuclease activity on distinct substrate binding and catalytic sites. The 3′- exonuclease activity appears to be dependent on divalent cations such as Zn(II) (Beese & Steitz, 1991). A structural role has been suggested for Zn(II) in aspartate carbamoyltransferase (Honzatko et al., 1982), another class II enzyme.

The present studies do not establish a specific role for metal ions in galactose-1-P uridylyltransferase, apart from being required for enzymatic activity. The fact that Co(II), Cd(II), and Mn(II) support activity 40–50% as well as Zn(II) or Fe(II) indicates that the metal ions may be more important in a structural role than as direct participants in catalysis. The X-ray crystallographic studies of galactose-1-P uridylyltransferase in our laboratory are at an advanced stage and will resolve this isssue (Wedekind et al., 1994).

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